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By Edward F. Mellon, Alfred H. Korn and Sam R. Hoover

Introduction

Many papers have been written on the water-absorbing characteristics of proteins in which the phenomena have been interpreted on the basis of the total protein molecule or the sum of all the polar groups in the molecule. Most of these interpretations have involved the significance of the various sections of the sigmoid curve obtained when the amount of water vapor absorbed is plotted against the relative humidity.

Recently Shaw[2] and Bull[3] by applying the Brunauer–Emmett–Teller[4] (B.E.T.) multilayer theory of adsorption of gases on solid surfaces obtained areas covered in the first layer which appear to be only fractions of the total area of the proteins when they are spread in thin films. Pauling[5] has shown that the number of molecules of water vapor absorbed in the first layer bears a relation to the total number of polar side chains in certain proteins. The various interpretations of the previous data are summarized in the review of McMeekin and Warner.[6] If the initial water absorbed is attached to specific polar groups and not absorbed in general on the surface of the molecule, it would be possible to decrease the amount of water vapor absorbed by converting the polar groups into groups with comparatively less capacity for hydrogen bonding. Such decreases should be proportional to the number of polar groups converted.

Observations on the benzoylation of proteins made by Goldschmidt and Schön[7] indicated that although benzoylation occurred on a number of reactive groups, the benzoyl groups were bound much more firmly to some groups than to others. The benzoyl group seemed to be more firmly attached to polar groups containing nitrogen than to those containing oxygen. Removal of benzoyl groups from the polar groups containing oxygen occurred very readily in weak alkali, leaving a product in which the benzoyl groups were predominantly attached to nitrogen. They also indicated that benzoyl groups attached to nitrogen did not make the protein alkali insoluble, whereas benzoyl groups attached to oxygen made the proteins insoluble in alkali.

It appeared possible that the velocity of reaction of benzoyl chloride with amino groups might be sufficiently greater than that with oxygen-containing groups to allow selective benzoylation. This has been accomplished by using limited amounts of benzoyl chloride in the reaction medium. A series of benzoylated casein samples has been prepared with varying amounts of free amino groups and a minimum of benzoyl groups attached to other polar groups. The free amino content of these benzoylated derivatives was readily determined by the method of Doherty and Ogg[8] for the amino groups of insoluble proteins.

This group of derivatives, in which there has been little change of the protein molecule except the covering of the free amino groups, makes it possible to study the effect of the amino group on the water-absorbing property of casein.

Experimental

Preparation of Benzoylated Caseins.—One liter of water was added to 220 g. of air-dried, high-grade, hydrochloric acid-precipitated casein, and the mixture was stirred for one hour to swell the casein granules. The suspension was diluted with four liters of water, and 1 *N* sodium hydroxide was added slowly with vigorous stirring. The *p*H was not allowed to go above 6.7, and at this *p*H about five hours were required to dissolve the casein. This solution, which was very turbid, was filtered with suction through a 1-cm. layer of paper pulp to remove any suspended fat globules and undissolved calcium phosphates and caseinates. The filtrate showed only a slight trace of turbidity. One normal alkali was added until the *p*H was 9.0. The desired amount of benzoyl chloride (Table I) dissolved in 100 ml. of anhydrous ether, was added slowly from a dropping funnel while the solution was subjected to vigorous agitation. One normal alkali was added continuously as required to keep the *p*H constant. About two hours was taken for the addition of the benzoyl chloride, and the solution was agitated for one half hour after the addition had been completed. The solution of benzoylated casein was then filtered with suction through a 1-cm. layer of paper pulp. The resulting solution, which showed only the faintest trace of turbidity, was diluted with an equal volume of water and precipitated slowly with 0.1 *N* hydrochloric acid while being agitated vigorously. The final *p*H ranged from 4.5 to 4.0, depending on the fraction of the amino groups which was expected to be covered. The finely divided suspended solid was allowed to settle, and the supernatant liquid was decanted. The residue was transferred to 250-ml. centrifuge bottles, and the solids were separated from the remaining liquid by centrifuging. The solid residue, which filled about one third of the bottle, was suspended in distilled water and recentrifuged (about ten times) until the supernatant no longer gave a test for chloride ion. The product was then washed in a similar manner with 50% alcohol and 95% alcohol, and three additional times with water to remove any traces of alcohol. The solid residue remaining after the last centrifuging was frozen in the centrifuge bottle and dried in the frozen state. The products dried to fine, fluffy, white powders, 95% of which passed through a 100-mesh sieve without grinding. The yield in all cases was about 200 g. The products were then spread out in thin layers and allowed to equilibrate with the moisture in the air for four days. All analyses reported in Table I were run on these equilibrated samples. The total nitrogen

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) Shaw, *J. Chem. Phys.*, **12**, 391 (1944).

(3) Bull, This Journal, **66**, 1499 (1944).

(4) Brunauer, Emmett and Teller, *ibid.*, **60**, 309 (1938).

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thirds of the cases it was less than 0.04 g., indicating good reproducibility in the experimental results.

In order to compare the water-absorbing properties of these benzoylated caseins containing various amounts of benzoyl substitution, it was assumed that the benzoyl group and the amino group substituted with benzoyl do not absorb water. If these assumptions are valid, the water absorbed per gram of total nitrogen is the significant variable to be considered, since it relates the water absorbed to the casein content of the material. These absorption figures for samples 1 and 4 are plotted as curves 1 and 2, respectively, against relative humidity in Fig. 1. The typical sigmoid-shaped absorption curve is obtained.

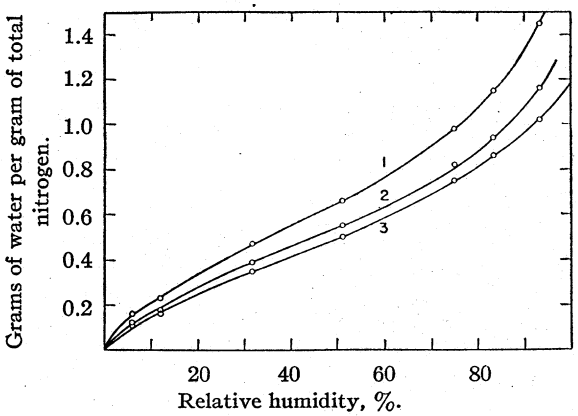


Fig. 1.—Absorption isotherms for casein (1), a benzoylated casein (2) and a hypothetical casein having no free amino groups (3).

When the average water absorption per gram of total nitrogen is plotted against the average amino nitrogen content per gram of total nitrogen (Fig. 2) a straight line is obtained. The straight lines were calculated by the method of least squares. The individual measurements of samples 1, 2, 3 and 4 were used; sample 5 was not used because it showed (Table I) a significant amount of non-amino benzoylation which may be on other hygroscopic groups. The slopes and intercepts of these lines are given in Table III. The intercepts when the amino content approaches zero should correspond to the water absorption due to the other groups of the protein. We have thus been able to distinguish between the water absorption occurring on the amino groups and that occurring on the other groups of the casein molecule. The intercepts for the 30.0° curves are plotted (curve 3) in Fig. 1. The sigmoid shape is still apparent.

TABLE III

WATER ABSORPTION BY AMINO AND NON-AMINO GROUPS OF CASEIN

Salt soln.	Relative humidity,[a] %	Figure II constants: Curve no.	Slope, g. H_2O / g. NH_2-N	Intercept, g. H_2O / g. N	Absorption on NH_2 groups in casein, % of total
		30.0°			
LiBr	5.9	1	0.87	0.106	33
LiCl	11.8	2	1.07	.162	29
$MgCl_2$	31.4	3	1.90	.350	25
$Mg(NO_3)_2$	50.9	4	2.73	.497	25
NaCl	75.1	5	3.82	.749	24
KCl	83.6	6	4.84	.858	25
KNO_3	93.3	7	7.29	1.011	30
		40.0°			
LiCl	11.0	8	0.97	0.157	27
$MgCl_2$	31.3	9	1.75	.321	25
$Mg(NO_3)_2$	48.3	10	2.38	.441	24
NaCl	74.7	11	3.75	.690	25

[a] Obtained from the linear plot of p *vs.* $1/T$ for all the available vapor pressure data of reasonable accuracy.

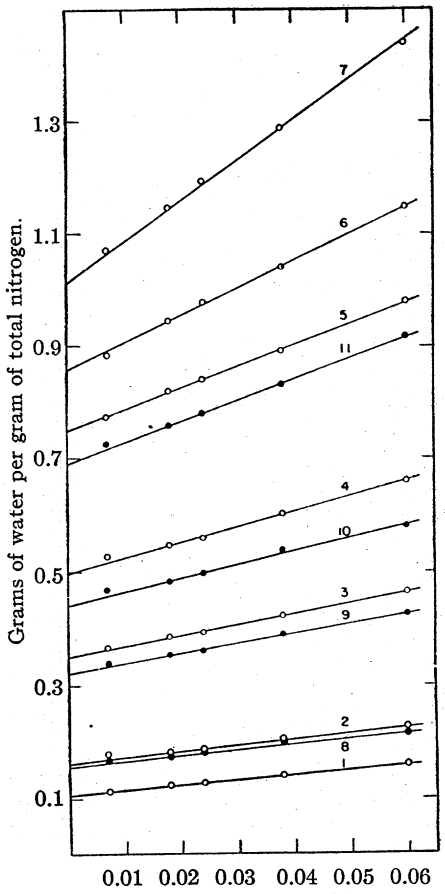


Fig. 2.—Water absorption by free amino groups in casein. The curves are identified by number in Table III.

The slope of the lines in Fig. 2 represents the grams of water absorbed per gram of amino nitrogen. These values have been plotted against relative humidity in Fig. 3 and may be interpreted as follows: Up to about 70% relative hu-

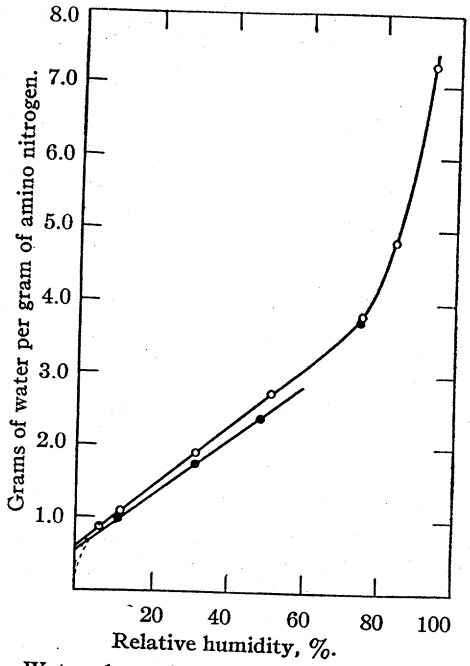
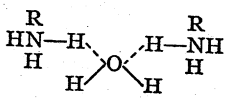


Fig. 3.—Water absorption isotherms for amino groups in casein: O, 30°; ●, 40°.

midity the graph is a straight line plot for which the equation by the method of least squares is

30.0° g. H_2O/g. NH_2—N = 0.0417 (% R. H.) + 0.60 (1)
40.0° g. H_2O/g. NH_2—N = 0.0378 (% R. H.) + 0.56 (2)

The intercepts correspond to 0.47 and 0.44 mole of water per mole of amino group, and appear to be identical within the limits of experimental error. We believe that this indicates the absorption of one molecule of water by two amino groups within the region between 0 and 6% relative humidity. Pauling[5] has postulated that there is a similar attachment of one molecule of water between two arginine side chain groups in salmin, and Bull[3] has indicated "a layer of water molecules between two coherent hydrophilic planes of protein molecules." He has suggested this as an interpretation of the a_1 term in the B.E.T. adsorption equation. Since the amino groups of casein are in the $-NH_3+$ form at the isoelectric point, it seems that the attachment of the water molecule must be between the positive hydrogen of the amino group and the oxygen of the water molecule.



The lysine content of casein accounts for more than 80% of the total free amino content. Consequently, if this assumption is correct, there must be a restriction on the position of the lysine residues within the casein molecule. One lysine epsilon amino group can approach another closely enough to hold a molecule of water between them only if the lysines are nearly opposite in adjacent peptide chains or laminae; or they are within the same chain either adjacent or separated by no more than two other amino acid residues which must have short side groups.

It is obvious that our data do not show the manner in which the curves of Fig. 3 approach zero absorption at zero relative humidity. It is, therefore, of value to present the alternative treatment where the graphs are assumed to follow a curved path in this region. These curves would be sigmoid in shape and the data could be treated by B.E.T. multilayer adsorption theory. According to this treatment, 1.05 moles of water per mole of amino group are required to form the first layer on the amino group, and the amino group accounts for 22% of the water absorbed in the monolayer on pure casein (sample 1), as calculated by the B.E.T. equation. This percentage agrees closely with the fraction of the total absorption due to the amino groups reported in Table III. The fact that the polar amino group, which constitutes less than 1% of the total weight of the protein, can account for about one quarter of the total water absorption indicates strongly that the water absorption of proteins occurs on specific sites (the polar groups) and that general surface adsorption plays a less important role. Cassie[11] has recently shown that such local sites for absorption do not necessarily have to be in the surface layer of the material; they can be distributed through the solid as in a hygroscopic gel.

The data can be further analyzed in terms of the B.E.T. treatment as follows. The statistical monolayer is complete at 18% R.H. The net heat of adsorption for this monolayer is 1.7 kcal. per mole. Similar analysis of curve 1, Fig. 1, gives 1.4 kcal. at 30° and 1.5 kcal. at 40° for the net heat of adsorption of the monolayer upon the pure casein sample. The heat of adsorption in the linear regoin of Fig. 3 can be directly calculated from the Clausius–Clapeyron equation. If we omit the intercepts (to eliminate the initial half mole of water) and assume that ΔH is independent of temperature, the heat of absorption for the straight line portion of Fig. 3 is 12.3 kcal. per mole of water absorbed from the vapor state. A similar calculation gives 1.85 kcal. per mole of water absorbed from the liquid state (the net heat of absorption). This heat of absorption is constant between 6 and 60% relative humidity and is of about the same magnitude as the heat of adsorption calculated by the B.E.T. theory for the first monolayer on the amino groups.

The linear relationship of Fig. 3 permits several explanations. The simplest is that there is a straight Henry's law solubility of water in the amino groups on the casein molecule. A more complicated explanation in terms of a series of equilibria wherein water molecules are added one at a time to the various hydrates is possible by using the mathematical treatment of Klotz.[12]

(11) Cassie, *Trans. Faraday Soc.*, **41**, 450 (1945).
(12) Klotz, *Arch. Biochem.*, **9**, 109 (1946).

These equilibria and their equilibrium constants have not been presented here because there appears to be a hysteresis phenomenon over part of the region concerned. A detailed study of this hysteresis is being made, and the effects which it may have on the mechanism of this linear adsorption will be presented in a later publication.

Above 70% relative humidity there is a rapid increase in the water absorbed on the amino groups. This is believed to be a condensation of water molecules on water molecules previously absorbed on the amino groups. This seems plausible because approximately 2.5 moles of water per amino group are absorbed at 60% relative humidity. This amount would be sufficient to saturate the hydrogen-bonding capacity of the amino group if the first molecule absorbed remains associated with two amino groups.

Table III shows the fraction of the total water absorption of casein which occurs on the free amino groups at each humidity studied. Previous treatments of the water-absorption of proteins have associated the effect of the various polar groups with some particular portion of the curve.[6] Our data show that the strongly polar amino group has its effect in all portions of the absorption isotherm.

Acknowledgment.—The authors gratefully acknowledge the suggestions of C. Roland Eddy regarding the free energy and heat of surface adsorption.

Summary

A series of benzoylated caseins has been prepared with varying amounts of free amino groups. Water-absorption studies on these samples have made it possible to distinguish between water absorbed on amino groups and water absorbed on the remaining groups of the casein.

The first step in the binding of water by the amino groups of casein seems to be a sharing of one molecule of water between two amino groups below 6% relative humidity. The B.E.T. treatment of the data, however, indicates a monolayer of one water molecule per amino group.

The second step is a linear increase in absorbed water with increase in relative humidity. Equations are presented for this increase between 0 and 60% relative humidity.

The third step is a rapidly increasing amount of absorption with increase of relative humidity and appears to be a condensation of water on water molecules already attached to the amino groups.

From 24 to 33% (depending on the relative humidity) of the water absorbed by casein is absorbed by the amino groups.